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14. ABSTRACT

To investigate how gain of function p53 mutants exert their oncogenic effects, protein expression was compared between a p53 null cell line stably expressing vector alone or the p53 gain of function mutants, p53-R175H and -R273H. One protein that was upregulated in cells expressing the p53 gain of function mutants control cell lines was identified by mass spectrometry as translocator of the mitochondrial membrane 50 (Tim50). p53-R175H and -R273H, but not WT p53, upregulated the luciferase activity of a Tim50 promoter construct. Loss of Tim50 expression also reduced the growth rate and survival from paclitaxel treatment in breast cancer cells that harbor p53-175H. Taken together, this data suggests that one pathway by which mutant p53 may upregulate cell growth and chemoresistance in breast cancer is through induction of Tim50 protein.

15. SUBJECT TERMS
Tim50, p53, Chemoresistance, Gain of Function

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1 INTRODUCTION

The tumor suppressor protein p53 is involved in the control of cell cycle progression, DNA integrity, and cell survival. Mutations in the p53 gene are some of the most frequent alterations in human cancers, with most of the mutations resulting in the expression of full-length mutant p53 proteins [1]. Mutant p53 proteins have altered transcriptional activity compared to WT, and often become stable and accumulate to high levels in tumor cells [2-7]. Expression of mutant p53 has been shown to impart increased cell growth [8-10] and chemoresistance to several chemotherapeutic agents in tumor cell lines [9, 11-13]. Mutant p53 expression has also been shown to contribute to metastasis in a mouse model for lung cancer [14, 15] and studies of human cancer indicate that the presence of p53 mutations is associated with poor prognosis in several types of tumors [16-19]. For example, in breast cancer, p53 mutation appears at a very high frequency in HER-2 positive tumors that are prone to metastasize [19]. This follows the GOF hypothesis, which predicts that mutations in the p53 gene not only destroy the tumor suppressor function of the WT protein but also impart increased oncogenicity [7, 20, 21]. While significant evidence indicates that mutant p53 contributes to oncogenesis, the exact mechanism of action of mutant p53 is still unclear [12, 22]. One hypothesis is that mutant p53 may act to alter gene expression. Several cell growth and survival related genes whose expression is altered by p53 GOF mutants have been identified by Deb and colleagues [9, 23, 24]. Other laboratories have also reported genes that are influenced by p53 mutants [25-28]. Many of the genes shown are associated with cell proliferation and tumor progression [6-8, 29, 30]. To further explore the mechanism of action of p53 GOF mutants, we performed large-scale mutant p53 immunoprecipitations coupled with mass spectrometry. One of the proteins we identified was Tim50 (Translocase of the inner mitochondrial membrane 50). Tim50 is one component of a large protein complex whose function is to import proteins into the inner mitochondrial matrix [31-33].

2 BODY

The aims of this study were to (1) Determine the contribution of Tim50 to the p53 GOF phenotype in breast cancer cells harboring mutant p53; (2) Examine whether Tim50 is a transcriptional target of mutant p53 and if Tim50 and mutant p53 contribute to each other's stabilization in breast cancer cells; (3) Determine if Tim50 expression is altered in human breast cancer cells and tumors and determine its relationship to p53 status and (4) Study the effect of Tim50 expression on mitochondrial protein import or mitochondrial membrane potential. Detailed methods are described in the appendix.

2.1 Objective 1: Determine the contribution of Tim50 to the p53 GOF phenotype in breast cancer cells harboring p53.

The presence of mutant p53 expression in breast cancer cells has been shown to increase chemoresistance from treatment with different therapeutic agents, and reduction of mutant p53 levels results in increased chemosensitivity [34, and unpublished results, Dr. Sumitra Deb]. As reported in the initial fellowship application, reduction of Tim50 protein expression resulted in a significant decrease in the survival of H-p53-R175H cells treated with paclitaxel but had no effect upon HC5 cells as measured by colony formation assays, Reduction of p53 protein expression, as expected, also reduced the survival of p53-R175H cells but not HC5 cells after treatment with paclitaxel.

To determine if Tim50 protein expression contributed to the chemoresistance of breast cancer cells expressing endogenous mutant p53, we performed Tim50 and p53 siRNA in SK-BR-3 (p53-R175H) breast cancer cells and measured colony formation after paclitaxel treatment. Consistent with the results obtained in H-p53-R175H cells, reduction of Tim50 or p53 protein expression in SK-BR-3 cells resulted in a significant decrease in colony formation after paclitaxel treatment (Figure 1).

These results strengthen the hypothesis that Tim50 is at least one of the causative agents for chemoresistance induced by p53 mutants.

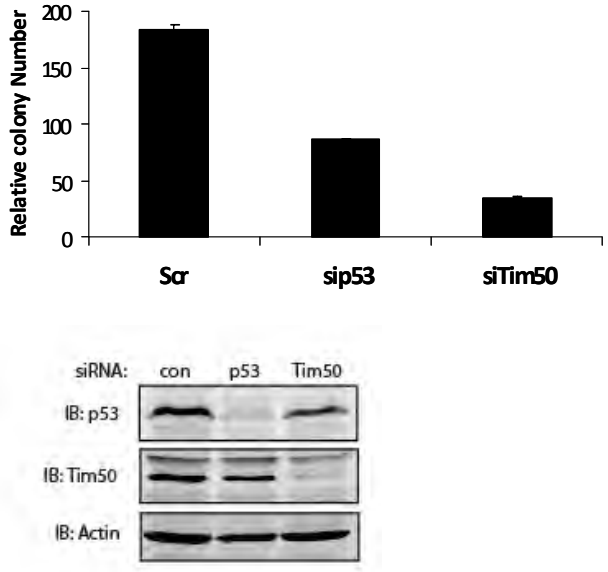

Figure 1. Loss of Tim50 Expression Reduces Survival of Mutant p53 Expressing Breast Cancer Cell Lines to Paclitaxel. (A) SK-BR-3 cells were transfected with scrambled siRNA or siRNA directed towards p53 or Tim50, and treated with paclitaxel (25 μM) for 48 hours and colony survival assays performed. **(B)** Western analysis of siRNA treated cells. Cell lysates were harvested 48 hours post-transfection and immunoblotted with the indicated antibodies. The data shown are from three independent experiments and colony numbers were adjusted to account for plating differences based on control plates treated with vehicle (DMSO). Relative colony numbers are shown.

In addition, it has previously been shown that the p53 mutant, R175H imparts a growth rate advantage when expressed in breast cancer cells [9]. To determine whether elevated Tim50 expression contributes to a growth rate advantage conferred by p53 GOF function mutants, Tim50 expression was reduced using siRNA in SK-BR-3 cells. The role of mutant p53 in growth rate enhancement in this cell line was similarly analyzed by p53 siRNA. Reduction of Tim50 protein expression significantly reduced the growth rate of SK-BR-3 cells (**Figure 2A**). Reduction of p53 protein expression, as expected, also reduced the growth rate of SK-BR-3 cells. The efficacy of siRNA in reducing Tim50 or p53 expression was confirmed by Western analysis (**Figure 2B**).

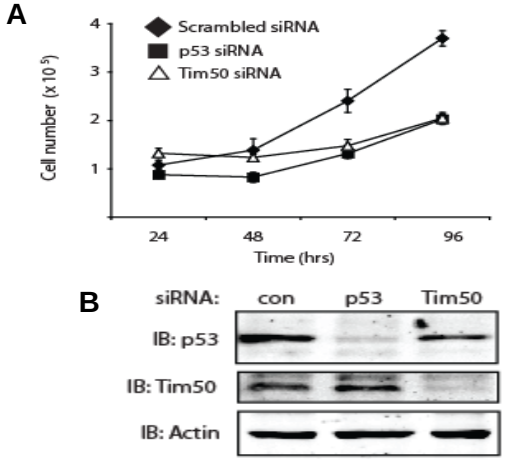

Figure 2. Loss of Tim50 Expression Impairs the Growth Rate of Mutant p53 Expressing Breast Cancer Cells. (A) SK-BR-3 cells were transfected with scrambled siRNA or siRNA directed against p53 or Tim50 and at the indicated times, cell numbers determined. Similar results were obtained in two additional experiments. **(B)** Western analysis of the siRNA treated cells. Cell lysates were harvested 48 hrs post transfection and immunoblotted with the indicated antibodies. The data shown are from three independent experiments and colony numbers were adjusted to account for plating differences based on control plates treated with vehicle (DMSO). Relative colony numbers are shown

2.2 Objective 2: Examine whether Tim50 is a transcriptional target of mutant p53 and if Tim50 and mutant p53 contribute to each other's stabilization in breast cancer cells.

To determine if mutant p53 could affect Tim50 expression through upregulation of the Tim50 promoter, a Tim50 promoter construct consisting of 1.97 Kb of the Tim50 promoter region cloned from genomic DNA (using information obtained in the NCBI database) and inserted into the pGL3 luciferase reporter plasmid (materials and methods) was. The Tim50 reporter construct, termed pGL3-Tim50, was then co-transfected along with WT p53 or the p53 mutants, R175H and R273H in H1299 cells. The p53 mutants, R175H and R273H, upregulated luciferase activity approximately 2.5 and 3 fold respectively, but WT p53, in contrast, inhibited Tim50 promoter activity (**Figure 3A**). Immunoblotting indicated that all p53 proteins were expressed in this assay (**Figure 3B**).

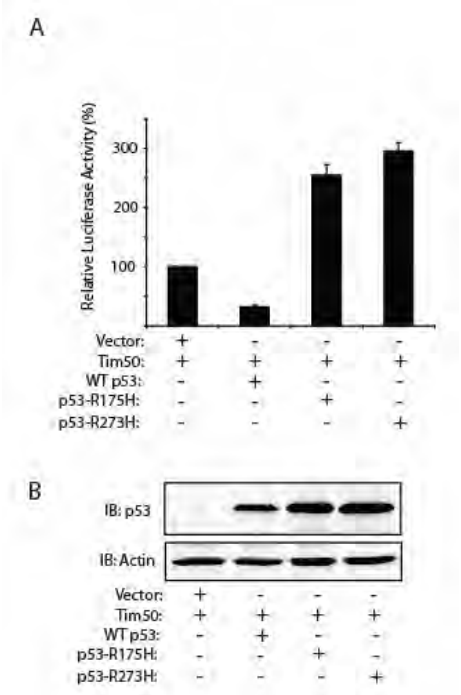

Figure 3. Mutant p53 up-regulates Tim50 promoter activity. (A) H1299 cells were transfected with a plasmid containing the Tim50 promoter region upstream of the luciferase reporter gene (pGL3-Tim50), the β-galactosidase control plasmid, and pCMVBam control plasmid (vector), or the indicated p53 plasmid for 48 hrs. After transfection, luciferase activity was detected using a luciferase reporter assay and values normalized to β-galactosidase values to control for transfection efficiency. * = significance $p < 0.05$. (B) Cell lysates from the transfections were blotted with the indicated antibodies. Similar results were obtained in two additional experiments.

These results suggest that the p53 mutants may operate directly at the promoter level of Tim50 to upregulate its expression. To explore the potential role for mutant p53 at the Tim50 promoter region, we measured the level of histone acetylation, as one indicator of chromatin structure, by Chromatin Immunoprecipitation (ChIP) assays in the absence and presence of mutant p53. ChIP assays were performed using anti-acetylated histone specific antibodies in combination with quantitative PCR directed against a region of the Tim50 promoter (bp 756-890). Using this approach, it was found that the Tim50 promoter region was quantitatively enhanced in the anti-acetylated histone immunoprecipitants from H-p53-R273H cells but not from HC5 cells (**Figure 4A**). No significant enrichment of the Tim50 promoter region was observed using control antibodies directed against human IgG.

The Tim50 promoter was also analyzed for potential transcription factor binding sites. Transcription factor binding sites on the Tim50 promoter were identified using TFSEARCH software (available on the World Wide Web at www.cbrc.jp/htbin/nph-tfsearch) with a cut-off threshold of 85%. Several putative sites were found for Ets-1, CREB and CBP. To determine if mutant p53 expression enhanced the presence of these transcription factors at the Tim50

promoter, ChIP assays using antibodies directed against Ets-1, CREB and CBP were performed. The Tim50 promoter region was greatly enhanced in anti-Ets-1, -CREB and -CBP immunoprecipitants from H-p53-R273H cells but not from HC5 cells (**Figure 4B, C and D**). Together these results indicate alteration of chromosome structure and an enrichment of several known transcription factors at the Tim50 promoter in the presence of mutant p53.

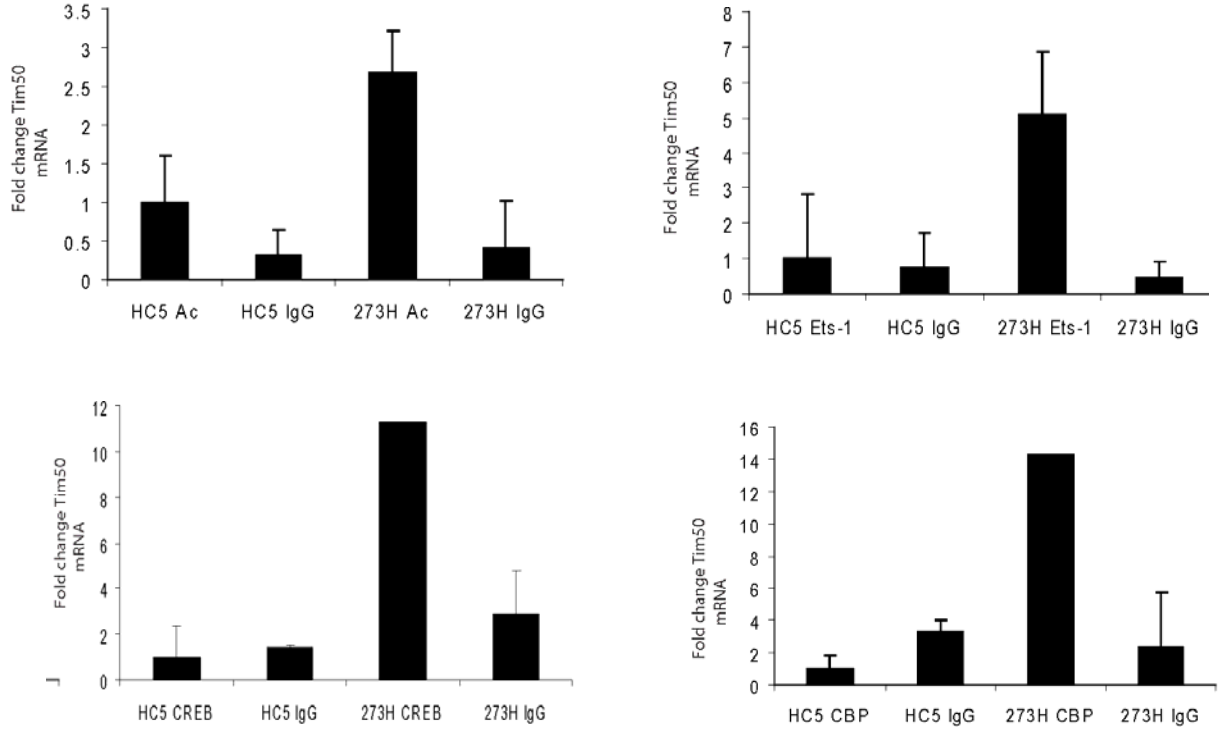

Figure 4. Mutant p53 expression enhances histone acteylation and transcription factor recruitment at the Tim50 promoter. (A) Chromatin immunoprecipitation (ChIP) assays were performed from HC5 or H-p53-R273H cells using antibodies specific for acetylated Histone H3 (AchH3) (A); Ets-1 (B); CREB (C); or CBP (D). The immunoprecipitates were assayed for the presence of the Tim50 promoter by quantitative PCR and Tim50 mRNA levels normalized to the internal standard, GAPDH mRNA. The normalized Tim50 mRNA level in the control cell line, HC5, was set to 1 and compared to the Tim50 mRNA levels in H-p53-R273H cells. The ChIP data were normalized to input DNA before immunoprecipitation. * = significance $p < 0.05$

Objective 3: Determine if Tim50 expression is altered in human breast cancer cells and tumors and determine its relationship to p53 status

2.2.1 Objective 3a. Determine the expression of Tim50 in breast cancer cell lines

To explore the relationship between mutant p53 and Tim50 protein expression further, several breast cancer cell lines were analyzed for p53 and Tim50 expression levels. Tim50 protein levels were elevated in MDA-MB-468 and SK-BR-3 total cell lysates which harbor the p53 mutants R175H and R273H respectively compared to MCF-7 and MCF-10A cells that express WT p53 (**Figure 5A**). Since WT p53 is expressed at low levels in MCF-10A cells and to determine if induction of WT p53 could alter Tim50 protein expression, we employed H1299

cells engineered to inducibly express WT p53 as previously described [34]. Induction of WT p53 protein expression in this cell line had no discernable effect onTim50 protein expression (**Figure 5B**).

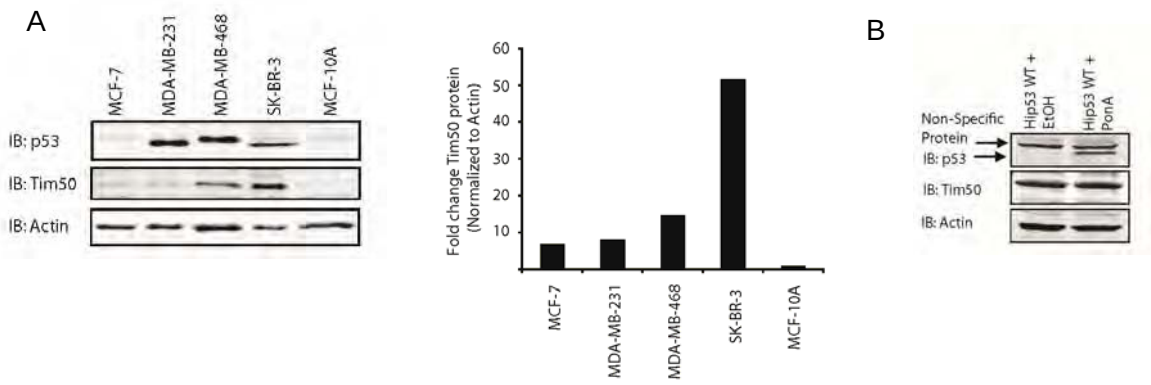

Figure 5. Expression of mutant p53 correlates with elevated levels of Tim50 mRNA and protein expression. (A) Total cell lysates from the indicated cell lines were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Equal protein amounts were loaded and Tim50 protein levels were normalized to actin. The relative amount of Tim50 protein compared to the level in MCF-10A cells is shown. The experiment was performed twice with similar results and a representative image is shown. (B) H1299 cells stably transfected with ecdysone-inducible WT p53 (Hip53 WT cells) were incubated in the presence (PonA) or the absence (EtOH vehicle) of 100 μM Ponasterone A for 24 hrs. Total cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

2.2.2 Objective 3b. Determine the expression of Tim50 in breast cancer patient tumor samples

No results were obtained pertaining to this objective.

2.3 Objective 4: Determine if Tim50 expression alters mitochondrial import or mitochondrial membrane potential

Using a novel assay utilizing a mitochondrial targeted GFP (mito-GFP) construct which only displays fluorescence upon import to the mitochondrial matrix, mutant p53 expression had no effect on mito-GFP import (**Figure 6**). However, downregulation of Tim50 by siRNA also had no effect on mito-GFP import (**Figure 6**). A different approach would need to be used to determine the effects of Tim50 expression and/or p53 status on mitochondrial import or mitochondrial membrane potential.

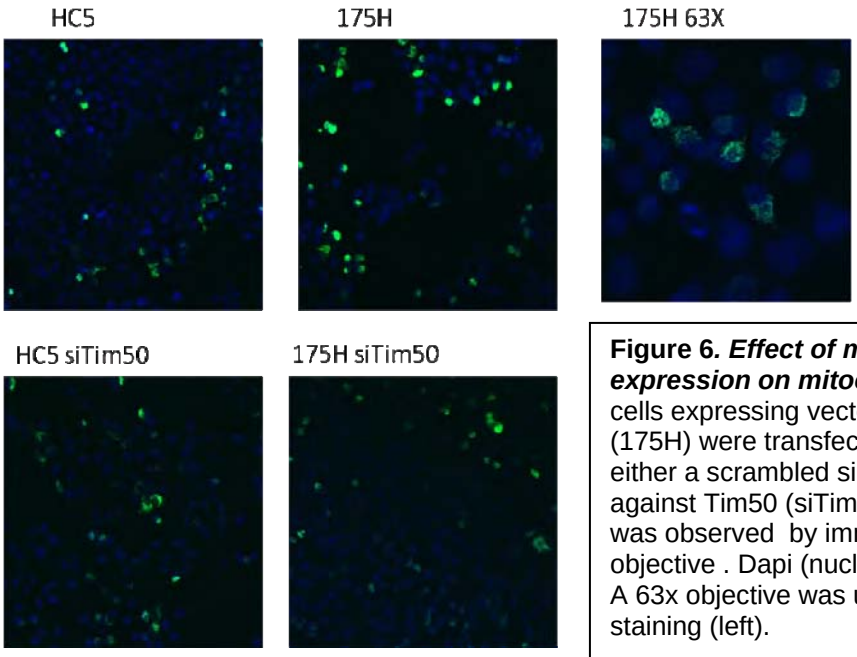

Figure 6*. Effect of mutant p53 and Tim50 expression on mitochondrial targeted GFP.* H1299 cells expressing vector (HC5) or mutant p53-R175H (175H) were transfected with mit-GFP (Green) and either a scrambled siRNA (control, *top*), or siRNAs against Tim50 (siTim50, *bottom*). Mit-GFP was import was observed by immunofluorescence using a 10x objective . Dapi (nuclei) staining is also shown (Blue). A 63x objective was used to confirm mitochondrial staining (left).

3 KEY RESEARCH ACCOMPLISHMENTS

- Inhibition of Tim50 by siRNA causes a significant reduction in chemoresistance in mutant p53-expressing breast cancer cells.
- siTim50 potently inhibits growth of human breast cancer cells that express GOF mutant p53.
- Mutant p53 could affect Tim50 expression through upregulation of the Tim50 promoter.

4 REPORTABLE OUTCOMES

Abstracts and Presentations:

Sankala, H., Harris, J.K., High, A., Mohanraj, L., Vaughan, C., Deb, S and Graves, P.R. Tim50, a Component of the Mitochondrial Translocator, Contributes to Cell Growth and Survival in Mutant p53 Expressing Cells. The 15th International p53 Workshop. 2010. University of Pennsylvania School of Medicine, Philadelphia. PA.

Sankala, H., Harris, J.K., Vaughan, C., High, A., Mohanraj, L., Deb, S and Graves, P.R. Upregulation of Tim50, by mutant p53 contributes to cell growth and chemoresistance. Massey Cancer Center Annual Research Retreat. October 28 2010. Virginia Commonwealth University, Richmond. VA.

5 TRAINING-RELATED PROGRESS

- Attended and presented data at bi-monthly joint group meetings.

- Passed the 'Preparing Future Faculty' course (GRAD 601) at Virginia Commonwealth University.
- Completed the Collaborative IRB Training Initiative (CITI) Basic Course in Biomedical Research as baseline entry into human subjects research at Virginia Commonwealth University.
- Prepared and submitted manuscript to Archives of Biochemistry and Biophysics:

    - **H Sankala,** C Vaughan, J Wang, S Deb, PR Graves. Upregulation of the mitochondrial transport protein, Tim50, by mutant p53 contributes to cell growth and chemoresistance.

Employment Opportunities:

Scientific Writer, Massey Cancer Center – participating in the development of clinical trial designs. Start date January 25th 2011.

6 CONCLUSIONS

Some mutations in the tumor suppressor protein, p53, have been termed 'gain of function' mutations because in addition to disabling the functions of WT p53, these mutations appear to impart additional functions to the protein that contribute to an increased oncogenic phenotype in cells in which they are expressed [8-13]. However, it remains unclear how p53 GOF mutants act to promote a GOF phenotype. To explore the mechanism of p53 GOF mutants, a proteomics study to identify mutant p53-specific interacting proteins was conducted. Using large scale immunoprecipitation of mutant p53 from H1299 cells expressing the p53 mutant, R175H, a co-precipitating protein that was not present in controls was detected Sequencing by mass spectrometry identified the protein as translocator of the inner mitochondrial membrane (Tim50). It was observed that elevated levels of Tim protein expression in several breast cancer cell lines that expressed endogenous levels of mutant p53, compared to cells expressing WT p53.

This study also suggests that the upregulation of Tim50 protein expression observed may be a result of mutant p53 acting either directly or indirectly at the Tim50 promoter. This conclusion is supported by the result that Tim50 transcriptional activity was upregulated by mutant p53 but not by WT p53 using a construct consisting of the Tim50 promoter. Moreover, ChIP assays also indicated that the chromatin structure of the Tim50 promoter was altered (as judged by histone acetylation) in the presence of mutant p53. The interaction of several transcription factors with the Tim50 promoter was also elevated in the presence of a p53 GOF mutant, R273H. Precedent for the interaction of p53 mutants with transcription factors was shown by the interaction of the p53 GOF mutant, D281G, with Ets-1 and the selective up-regulation of endogenous human MDR1 expression [35]. Taking together, these findings strongly suggest that the *Tim50* gene is a transcriptional target of p53 GOF mutants. While the p53 utilized in these studies, R273H, was found to behave qualitatively in a similar manner to p53-R175H with regard to Tim50 activation and expression, it should be noted that p53-R175H and p53-R273H represent different classes of p53 mutants: p53R-175H is a conformational mutant, whereas p53-R273H is a DNA contact site mutant [36]. These conclusions may therefore be relevant to a broad range of p53 mutants implicated in breast cancer. Further studies in breast cancer will be necessary to determine if there is a correlation between mutant p53 status, Tim50 expression, and tumorigenicity.

What might be the role of increased Tim50 protein expression in the action of p53 GOF mutants in breast cancer? Tim50 forms part of a large protein complex that functions to import proteins with mitochondrial presequences into the mitochondrial matrix [33, 37]. This includes all mitochondrial matrix and a number of inner mitochondrial membrane proteins required for normal mitochondrial function. Specifically, Tim50 is thought to facilitate transfer of proteins from the outer membrane of the inner mitochondrial space across the inner mitochondrial membrane. In addition to participating in transport of proteins, it is thought that Tim50 maintains the permeability barrier of mitochondria by closing the translocation pore [33]. Indeed loss of Tim50 results in the loss of mitochondrial membrane potential (MMP) in yeast [33] and flies [38]. Perhaps as a result of the defect in mitochondrial function, Tim50 null flies were also found to be reduced in size and cells derived from the flies showed a proliferation defect [38]. Thus, Tim50, through its essential role in the transport or proteins into the mitochondrial matrix, has been shown to play an active role in the modulation of growth and development [38].

How might upregulation of the Tim50 protein contribute to the increased survival from paclitaxel treatment observed in breast cancer cells expressing GOF mutant p53? Paclitaxel is used to treat breast cancer, after combination anthracycline and cyclophosphamide therapy. Paclitaxel is given for early stage and metastatic breast cancer and is also given as neoadjuvant treatment. Paclitaxel treatment has been shown to induce apoptosis in breast cancer cells [39-40] whereas reduction of Tim50 expression was shown to increase the sensitivity of human cell lines to death stimuli by increasing the rate of cytochrome C release from mitochondria [41]. For example, it was shown that loss of Tim50 expression by siRNA enhanced cytochrome C release and cell death after treatment of HEK293T cells with UV or staurosporine [42]. Therefore, it is conceivable that *increasing* the level of Tim50 protein may confer a greater resistance to cytotoxic agents such as paclitaxel.

In summary, this study shows for the first time that a protein required for the import of proteins into the mitochondrial matrix, Tim50, is upregulated in breast cancer cells that harbor p53 GOF mutants. The data also suggests that upregulation of protein import into the mitochondria or perhaps maintenance of mitochondrial membrane potential may confer a selective growth or chemoresistance advantage to breast cancer cells. In this context, these findings suggest that the upregulation of the Tim50 protein by mutant p53 may be a survival strategy for breast tumors that harbor p53 mutations and it may allow for the development of unique strategies centered around protein import into the mitochondria that could allow for the selective targeting of cancer cells. Alternatively, if Tim50 upregulation is a widespread mechanism for breast cancer cell survival, measurement of Tim50 expression in breast cancer cells may be a valuable biomarker for oncogenic potential and tumor development.

Overall the results suggest that one pathway by which mutant p53 may upregulate breast cancer cell growth and chemoresistance is through induction of Tim50 protein expression.

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8 APPENDIX

8.1 Methods

8.1.1 Cell culture

SK-BR-3, MDA-MB-231, MDA-MB-468, MCF-7 and H1299 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% (v/v) fetal bovine serum. MCF-10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 µg/mL EGF, 0.5 µg/ml hydrocortisone, 0.1 µg/ml choleratoxin, and 10 µg/ml insulin. Cells were maintained at 37 ^{o}C in an atmosphere of 5 % CO_2. Stable H1299 cell lines that expressed vector alone (designated HC5), or p53-R175H or p53-R273H (designated H-p53-R175H or H-p53-R273H respectively) were generated as previously described [23] and maintained in 400 µg/ml gentamycin. The H1299 p53-WT inducible cell line (Hip53) was generated as previously described [34]. p53 in this cell line was induced for 24 hrs with 100 µM Ponasterone A (Invitrogen, Carlsbad, CA)

8.1.2 Plasmid construction and RNA interference

To create a Tim50 promoter construct upstream of luciferase, a 1.97 kb region of the Tim50 gene promoter was amplified by PCR with the following primers: 5'-CCAAGCTTCGAGAGAGACCAAAGGCATC-3' (with Hind III site underlined) 5'-CCGGGTACCCTCGTTTCTCACTCAAGCCCT-3' (with Kpn I site underlined). The PCR product was digested with Hind III and Kpn I and ligated into the pGL3 vector (Promega, Madison WI) upstream of the luciferase reporter gene. This construct was designated as pGL3-Tim50. The correct sequence of the Tim50 promoter was verified by sequencing (Molecular Cloning Laboratories, San Fransisco, CA). Approximately 3 x 10^6 cells were transfected for 48 hrs or 96 hrs with either a scrambled siRNA (control), or siRNAs against Tim50 or p53. The siRNA sequences were as follows: Scrambled: 5′-CAUGUCAUGUGUCACAUCACTT-3′; Tim50: 5′-CGAACGGUGCUGGAGCACU-3′ and p53: 5′-GCAUGAACCGGAGGCCCAU-3′. Cells were transfected by electroporation using the Amaxa Nucleofection kit according to the manufacturer's instructions (Amaxa, Koeln,

Germany) using appropriate conditions for each cell line. The efficacy of siRNA treatment was confirmed by Western analysis.

8.1.3 Colony survival assays

Approximately 3 x 10^6 cells were transfected for 48 hrs with scrambled, Tim50 or p53 siRNA as described above. Cells were plated at a density of approximately 1 x 10^4 cells per 100-mm plate and treated with 25 nM paclitaxel for 48 hrs. After treatment, cells were washed with PBS, and fresh media was replaced. The cells were allowed to form colonies with periodic changes of media for a period of ~2-3 weeks. Colonies were fixed with 100% methanol, stained with 0.02% methylene blue and counted as previously described [9]. Control samples were treated with drug vehicle, DMSO, to measure plating efficiency.

8.1.4 Cell growth assays

Approximately 3 x 10^6 cells were transfected for 48 hrs with scrambled, Tim50 or p53 siRNA as described above. Cells were plated at a density of 1 x 10^3 or 1 x 10^5 cells per 60-mm plate respectively and after 24, 48, 72 and 96 hrs, cells were harvested and counted in a Coulter Counter.

8.1.5 Western blot analysis

Cells were lysed in mammalian cell lysis buffer which contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail (Roche, Nutley, NJ). Equal amounts of protein were resolved by SDS/PAGE (8% or 12% gels) and transferred to nitrocellulose membranes. The primary antibodies used for immunoblotting were all used at 1:1000 dilution and included: Tim50 (#IMG-3375, Imgenex, San Diego, CA), actin (#1615, Santa Cruz Biotech, Santa Cruz CA), and p53 (#9282, Cell Signaling, Danvers, MA). A monoclonal p53 antibody was also used, prepared as previously described [36]. Primary rabbit polyclonal antibodies were detected using the following secondary antibodies (all at 1:7000 dilution): IRDye800-conjugated affinity-purified rabbit anti-IgG antibody (Rockland Immunochemicals, Gilbertsville, PA), Alexa Fluor® 680-conjugated goat anti-mouse IgG antibody (Molecular Probes, Invitrogen, Carlsbad, PA) and Alexa Fluor® 680-conjugated rabbit anti-goat IgG antibody (Invitrogen, Carlsbad, CA). Proteins were visualized using the Odyssey system (LI-COR) and where indicated, relative amounts of immunoreactive protein in each band were determined by densitometric analysis and normalized to the level of actin.

8.1.6 Quantitative PCR

Q-PCR was performed as described previously [9]. Briefly, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). The quality of RNA was checked by 1.2% agarose Tris-borate-EDTA gel electrophoresis and cDNA synthesized using the SuperScript II kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Q-PCR was conducted using a LightCycler system (Roche, Nutley, NJ) as described previously [9]. Primers were designed using OLIGO 5 software (Molecular Biology Insights, Cascade, CO). Reactions were performed in triplicate utilizing SYBR green dye (Invitrogen, Carlsbad, CA) using the following primers: Tim50, 5'-CCGTACTACCAGCCACCCTA-3' and 5'-TGGGGGTCCACACTATCAAT-3', Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5'-GTCAACGGATTTGGTCGTATT-3' and 5'-GATCTCGCTCCTGGAAGATGG -3'. Tim50 promoter, 5'-GCGTTGGTGGTGGCGAGGTA-3' and 5'-AGCGGAGGCGGGGAAGG-3'.

8.1.7 Luciferase reporter assays

Cells were triply transfected with 100 ng of control β-Gal plasmid, 200 ng of the Tim50 promoter-luciferase reporter construct (pGL3-Tim50) and 1500 ng of vector only (pCMVBam) or WT p53, p53-R175H, or -R273H in pCMVBam for 48 hrs [8]. After transfection, cells were harvested and luciferase activity measured using the Promega luciferase assay kit (#E1500, Promega, Madison, WI) according to the manufacturer's instructions. Cell extracts were normalized to each other based on total protein concentration and Luciferase activity detected using a Luminometer from Turner Designs. Total luciferase activity was normalized with respect to β-Galactosidase activity measured using the Promega β-Galactosidase Enzyme Assay System (#E2000, Promega, Madison, WI) according to the manufacturer's instructions.

8.1.8 ChIP analysis

Exponentially growing H1299 cells expressing vector (HC5) or mutant p53-R273H (3×10^6) were cross-linked with 1% formaldehyde for 15 min and the reaction stopped by addition of glycine to a final concentration of 0.125M. Cells were collected and washed once with PBS. Pellets were resuspended in RIPA buffer (150 mM NaCl, 50mM Tris pH 8, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) and then sheared by multiple passages through a 27.5 gauge needle, followed by 25 min of sonication on ice to induce chromatin fragmentation. Following centrifugation, the protein content of the supernatents was determined and equal amounts used for immunoprecipitation overnight at 4^oC with gentle tilting with either anti-Actetyl-Histone H3 (#17-615, Milipore, Billerica, MA), anti-Ets-1 (#sc-350, Santa Cruz Biotech, Santa Cruz CA), anti-CREB (#sc-186, Santa Cruz Biotech, Santa Cruz CA) or anti-CBP (#sc-369, Santa Cruz Biotech, Santa Cruz CA), or IgG as a control (Normal rabbit IgG, Milipore, Billerica, MA). Immune complexes were captured the following day with protein A-agarose. The immunoprecipitate was pelleted and washed once with RIPA buffer, once with a high salt wash (500 mm NaCl, 50 mm Tris–HCl, pH 8, 0.1% SDS, 1% NP-40), twice with a LiCl wash (250 mM LiCl, 50 mM Tris–HCl, pH 8, 0.5% sodium deoxycholate, 1% NP-40) and twice with TE buffer. The antibody–DNA complexes were eluted with elution buffer (20% SDS, 10 mM DTT, 100 mM $NaHCO_3$) and the crosslinking was reversed by incubation at 65°C overnight. pGEM(3z)f-(3 ng) was added to each sample to act as an internal control and the DNA was then ethanol precipitated. Samples were then dissolved in TE and RNAse (10 mg/ml) and treated with proteinase K (20 mg/ml). Proteins were removed by phenol–chloroform extraction and the DNA isolated by ethanol precipitation. The DNA pellets were dissolved in TE and Q-PCR was carried out as described above.

8.1.9 Immunofluorescence

H1299 cells expressing vector (HC5) or mutant p53-R175H (175H) were grown on 12-mm-diameter round coverslips (VWR International) in 12-well plates and transfected with mitochondrial targeted-GFP and either a scrambled siRNA (control), or siRNA against Tim50 (siTim50). After 48h cells were washed twice with PBS, fixed for 15 min at room temperature with 4% (w/v) paraformaldehyde in PBS, washed twice with PBS containing 10 mM glycine (pH 7.4) and incubated for 1 h in blocking/permeabilization buffer [10 mM glycine, 1% BSA and 0.5% Triton X-100 in PBS (pH 7.4)]. Cells were washed three times and coverslips mounted on to glass slides (Fisher) using the ProLong® Antifade Kit (Molecular Probes) and examined with a Zeiss LSM 510 laser-confocal microscope using a ×10 or ×63 oil-immersion objective lens.